Volume 17 Number 12 1989 Nucleic Acids Research

Regiospecific inhibition of DNA duplication by antisense phosphate-methylated oligodeoxynucleotides

Harold M.Moody, Marcel H.P.van Genderen, Leo H.Koole, Henk J.M.Kocken, Emmo M.Meijer and Henk M.Buck

Department of Organic Chemistry, Eindhoven University of Technology, PO Box 513, 5600 MB Eindhoven, The Netherlands

Received March 3, 1989; Revised and Accepted May 15, 1989

ABSTRACT

A new synthesis route for long phosphate-methylated oligodeoxynucleotides is described, which were used as antisense inhibitors of the DNA replication. Phosphate-methylated oligomers hybridize more strongly with natural DNA than their natural analogues, due to the absence of electrostatic interstrand repulsions. Compared with phosphate-ethylated and methyl phosphonate systems, phosphate-methylated systems are preferable as antisense DNA, which was concluded from the high T_m values and sharp melting transitions of duplexes of phosphate-methylated and natural DNA. By using the Sanger dideoxy technique, it was shown that a complementary phosphate-methylated 18-mer can effectively and site-specifically block the DNA replication process at room temperature.

INTRODUCTION

In recent years, considerable attention has been given to antisense hybridization arrest techniques for retardation of replication and transcription processes (1-3). Originally, the work of Zamecnik et al. (4) showed that a large excess of a natural antisense oligonucleotide can block the replication of Rous sarcoma virus in chick fibroblast cells. More effective techniques were developed using DNA analogues that are not susceptible to enzymatic degradation, such as oligomers with thiophosphate linkages (5-9) and neutral DNA systems with phosphate triester (5, 10-16) or phosphonate linkages (17-19). Especially the neutral oligonucleotides are attractive because of their lipophilic character, which allows easy transport through cellular membranes, and because of the strong hybridization with natural DNA as a result of the elimination of phosphate-phosphate electrostatic repulsions.

Recently, we have shown that phosphate-methylated oligodeoxynucleotides are preferable as antisense DNA, since they display stronger hybridization than methyl phosphonate or phosphate-ethylated systems (12,13). The replacement of oxygen by methyl in methyl phosphonate oligodeoxynucleotides disturbs the conformation around the P-$O_{3'}$ and P-$O_{5'}$ linkages, due to stereoelectronic

factors. Therefore, longer (>8 nucleotides) methyl phosphonate systems do not readily adopt a helical geometry, resulting in poor hybridization with natural DNA. Indeed, it is found that methyl phosphonate 21-mers have a low antisense activity, compared to smaller systems (1). Phosphate-ethylated oligodeoxynucleotides have the disadvantage that steric interactions allow stable hybrids with natural DNA only for the Sp configuration (ethyl group pointing outward). Therefore, for longer oligomers only a minute fraction of the diastereomers is active as antisense agent. For phosphate-methylated oligomers, no effect of the phosphorus configuration is seen on the strength of hybridization with natural DNA, due to the smaller size of the methyl group. Therefore, all diastereomers are equally effective.

In this paper, we report a new synthesis route for longer phosphate-methylated oligodeoxynucleotides, and study their use as antisense replication blockers. Usually in oligonucleotide synthesis the exocyclic amino groups of the A, C and G bases are protected with acyl groups (for instance benzoyl and isobutyryl), which are removed at the end of the synthesis by treatment with concentrated ammonia at 55 °C. Under these conditions, however, phosphate-methylated internucleoside linkages are cleaved as a result of the nucleophilic character of aqueous ammonia. We therefore recently applied the 9-fluorenyl methoxycarbonyl (Fmoc) group as a base-protecting moiety (20-22). It can easily be removed by β-elimination with a non-nucleophilic base such as triethylamine, while leaving the phosphate triesters intact. With the standard phosphite triester protocol, we prepared phosphate-methylated trimers and tetramers stepwise in solution. Longer oligomers (10 - 20 nucleotides) that can act as sequence specific antisense DNA, have to be prepared on a solid support however. Because all commercially available linkers between solid phase and DNA have to be cleaved with aqueous ammonia, we combined in this work the Fmoc strategy with the standard automated DNA synthesis as follows: (i) Natural oligomers are prepared on a solid support at 10 μmol scale, and afterwards all exocyclic amino groups of the bases are protected in solution with Fmoc; (ii) Methylation of the phosphate groups is performed as described by Miller *et al.* (23) with p-toluenesulphonyl chloride and methanol; (iii) Complete deprotection of the bases with triethylamine/pyridine yields the aimed-for phosphate-methylated oligodeoxynucleotide.

In order to test the effectiveness of phosphate-methylated systems for antisense hybridization arrest techniques, we studied the *in vitro* replication of the *E.coli* pabB gene, inserted in the M13mp18 phage. This circular single-stranded DNA is routinely used in our laboratory for *in vitro* mutagenesis

experiments, and is a well-defined model system. It will be shown that replication of the pabB gene can be stopped at a chosen location with an appropriate phosphate-methylated 18-mer at 20 °C. This indicates the effectiveness and specificity of the phosphate-methylated blockers.

EXPERIMENTAL

DMF was distilled under reduced pressure and stored on 4 Å molecular sieves. Anhydrous pyridine and anhydrous 2,6-lutidine were prepared by refluxing with solid KOH, followed by distillation, and were stored on 4 Å molsieves. Triethylamine was also dried with KOH and then distilled from P_2O_5. Methanol was stored in contact with 3 Å molsieves for several days prior to use.

General procedure for phosphate-methylation

After the natural oligodeoxynucleotide is prepared with a DNA synthesizer, the temporary protection of the bases with Fmoc and the methylation of the phosphate groups are accomplished in solution. By adding a large excess of 9-fluorenyl methoxycarbonyl chloride (FmocCl), cytosine and guanine react with one, and adenine with two molecules FmocCl; the 3' and 5' hydroxyl functions are protected too. Afterwards the excess of FmocCl is removed with methanol, and methylation of the phosphate groups is performed according to the procedure of Miller *et al.* (23) with methanol and p-toluenesulphonyl chloride in N,N-dimethylformamide and 2,6-lutidine. Finally, the bases and hydroxyl groups are deprotected in a 1:1 (v/v) mixture of pyridine and triethylamine. In this way phosphate-methylated d(GGA.ATC), d(AGC.CTG.AC), d(GGA.ATC.CTG.CAG) and d(CAC.TCA.CCC.ATG.AAC.AGC) were prepared, with overall yields of 37%, 31%, 33% and 37%, respectively. As an example of the synthesis of longer oligomers via the combination of solid phase and solution synthesis, the detailed preparation of phosphate-methylated d(CAC.TCA.CCC.ATG.AAC.AGC) is given hereafter.

Preparation of d(CAC.TCA.CCC.ATG.AAC.AGC)

The preparation of the natural octadecanucleotide d(CAC.TCA.CCC.ATG.AAC.AGC) was performed on an Applied Biosystems 381A DNA synthesizer according to the phosphite triester method. The synthesis was started with a solid support of controlled pore glass loaded with 10 µmol of 2'-deoxy-4-N-benzoylcytidine. Each coupling was accomplished by adding the appropriate base-protected 5'-O-dimethoxytrityl-2'-deoxynucleoside β-cyanoethyl phosphorusamidite (A and C bases were protected with a benzoyl group, the G base with an isobutyryl group). Coupling efficiencies were

determined by monitoring the release of the dimethoxytrityl cation (99% average). Afterwards the column was treated 5 times for 20 minutes with concentrated ammonia (Merck art. 5426, ammonia solution 32%, extra pure) at room temperature, leading to cleavage from the solid support and deprotection of the phosphate groups. The resulting solution of DNA in ammonia was held at 55 °C for 10 h in order to ensure complete deprotection of the A, C and G bases. After evaporation of all volatile matters, the residue was dissolved in 2.0 ml H_2O. Finally the DNA was precipitated at -20 °C, after adding of 18.0 ml ethanol and 0.20 ml 3 M NaAc (adjusted to pH = 5.6 with concentrated HCl). Yield: 9.5 µmol (95%, 56 mg), determined by measuring the UV extinction at 260 nm.

Preparation of phosphate-methylated d(CAC.TCA.CCC.ATG.AAC.AGC)

56 mg (9.5 µmol) d(CAC.TCA.CCC.ATG.AAC.AGC) was coevaporated with anhydrous pyridine (3 x 10 ml) and suspended in 15.0 ml pyridine, and 200 mg (0.76 mmol) 9-fluorenylmethoxycarbonyl chloride was added. The pale yellow suspension turned clear and yellow after 15 h stirring and was then quenched with 30 ml dry methanol. After 30 minutes the mixture was concentrated *in vacuo* and the pyridine was removed by coevaporation with toluene (3 x 10 ml) and chloroform (4 x 10 ml). The residue was dissolved in a solution containing dry N,N-dimethylformamide (4.8 ml), dry methanol (2.4 ml) and dry 2,6-lutidine (1.95 ml). The solution was treated with p-toluenesulphonyl chloride (0.87 g) for 15 h at room temperature and with water (1 ml) for 30 minutes at 0 °C. The solvents were then evaporated with ethanol (30 °C, 0.1 mm Hg). The residue was dried by repeated evaporation with anhydrous pyridine and stirred in 20 ml of a mixture of pyridine and triethylamine (1:1, v/v) for 3 h. During the deprotection of the bases, the solution turned from pale yellow to yellow-orange. The mixture was evaporated and the residue coevaporated with toluene (3 x 10 ml) and chloroform (4 x 10 ml), and the resulting oil was treated with methanol (10 ml) which gave a white solid and an orange solution. The solid was washed twice with methanol, the washings and the orange solution were combined and concentrated, and purified by preparative thin layer chromatography on Merck silica gel 60 F254 plates (2 mm thick). Eluent: methanol; R_f = 0.1 . The product was extracted from the silica gel by shaking with water (2 x 25 ml) during 20 h. The water and the silica gel were separated by centrifugation (15,000 g) and concentration of the supernatant *in vacuo* yielded 21 mg (37%) phosphate-methylated d(CAC.TCA.CCC.ATG.AAC.AGC).

Spectroscopy

NMR spectra were recorded on a Bruker AC 200 spectrometer at 200 MHz for ^1H and at 80.9 MHz for ^{31}P. Optical measurements were made on a Perkin Elmer

124 double beam spectrophotometer, using 10-mm cuvettes. Absorbance was recorded at 260 nm as a function of temperature. All duplexes were formed in 1:1 mixtures of a strand with its complement. All samples were pre-melted for 2 min at 70 °C to destroy secondary structure and then allowed to cool to room temperature slowly.

Sequencing

Sequencing was essentially performed as described by Sanger (24). DNA polymerase I (Klenow fragment), which was purchased from Pharmacia as a solution in 50 mM potassium phosphate buffer (pH = 7.0), 0.025 mM dithiotreitol and 50 % glycerol, was diluted just before use from 7 u/µl to 1 u/µl with the sequencing buffer (10 mM Tris/HCl, pH = 8.0 and 5 mM $MgCl_2$). The template for the sequencing reaction was the *E.coli* pabB gene, inserted in M13mp18 phage, using polylinker sites EcoRI and Hind III. The 18-mer primer d(AGT.AAT.CAC.AGC.GGG.AGA) was synthesized on an Applied Biosystems 381A DNA synthesizer at 0.2 µmol scale. A mixture of 1 µl 10x sequencing buffer (100 mM Tris/HCl, pH = 8.0, 50 mM $MgCl_2$), 1 µl primer (1.0 pmol/µl), 1 µl template (1.2 pmol/µl), 1 µl phosphate-methylated d(CAC.TCA.CCC.ATG.AAC.AGC) (62 pmol/µl) and 6 µl water was incubated at 70 °C for 2 min and then allowed to cool slowly to room temperature for at least 45 min in fraction collector tubes, in order to achieve annealing of primer and phosphate-methylated DNA (blocker) to the template. In the control experiment water was used instead of phosphate-methylated DNA. 1 µl α-^{32}P-dATP (10 µCi, 3000 Ci/mmol), 1 µl Klenow DNA polymerase I (1 u/µl) and 0.5 µl H_2O were mixed, added to the template-primer-blocker complex solution and 3 µl of this solution was transferred into 2 µl dideoxy-mix, either d/ddTTP, d/ddGTP, d/ddCTP or d/ddATP. After 15 minutes at room temperature 1 µl dNTP chase solution was added (containing all four dNTP's, 0.125 mM of each) and the sequencing reaction continued for another 15 minutes. Concentrations at the start of the reaction were: template: 0.058 pmol/µl, primer: 0.048 pmol/µl, phosphate-methylated DNA: 3.0 pmol/µl and Klenow DNA polymerase I: 0.048 u/µl = 0.090 pmol/µl.

Polyacrylamide gel electrophoresis and autoradiography were performed according to standard procedures.

Determination of the relative synthesis activity via measurement of the sequencing pattern densities

The optical density of the sequencing bands after autoradiography was measured with a radiological densitometer (Nuclear Enterprises Ltd, model 2526). The absorbance was determined in the middle of each spot and related to

the absorbance of the unexposed film (zero density). In this way the relative synthesis activity of the Klenow DNA polymerase I along the template strand can in principle be determined. If all (specific and non-specific) stops are taken into account however, a plot of the total stops as a function of position on the template would be obtained. Hence, only (specific) stops as a result of incorporation of a dideoxynucleotide were measured, not the (non-specific) stops as a result of direct affection of the DNA polymerase. Each measured spot was related to the corresponding spot on the reference sequencing film (water added instead of phosphate-methylated blocker). The relative synthesis activity as a function of template position was obtained by taking the ratio of the absorbance of a spot on the blocker sequencing pattern and the corresponding spot on the control sequencing pattern.

RESULTS AND DISCUSSION

Synthesis of phosphate-methylated oligodeoxynucleotides

One of the aims of this work was to develop a new synthesis route for phosphate-methylated oligodeoxynucleotides with an arbitrary length and base sequence. In order to test the new strategy, several phosphate-methylated oligodeoxynucleotides of various lengths were synthesized (*vide supra*). The products were characterized with ^{31}P and ^{1}H NMR and optical measurements (UV). Because the methylation procedure with methanol and p-toluenesulphonyl chloride is not stereospecific, each phosphate-methylated sequence consists of a mixture of 2^n diastereomers (n = the number of phosphates), which makes the characterization with NMR more difficult. Figures 1A and 1B, for example, show the ^{31}P NMR spectra of the octadecamer d(CAC.TCA.CCC.ATG.AAC.AGC) and its phosphate-methylated analogue, respectively. From comparison of the two spectra it can be concluded that methylation of the phosphate groups does not change the ^{31}P NMR shifts dramatically. In the ^{31}P spectrum of the natural octadecamer separate peaks are identifiable. The spectrum of the phosphate-methylated 18-mer on the other hand is one single broad resonance, due to the complexity of the diastereomeric mixture ($17x2^{17} = 2.2x10^6$ signals). The ^{1}H NMR spectrum of this phosphate-methylated 18-mer provides more detailed information on phosphate methylation via the phosphate methyl resonances (Figure 2). The $POCH_3$ signals are complex too, but they are clearly distinct from all other proton resonances, except the $H_{5'/5''}$ of the 5'-end nucleotide. Comparison of the surface area of the $POCH_3$ and 5'-end $H_{5'/5''}$ resonances in the 3.7-3.4 ppm region with other proton resonances, for example the combined $H_{1'}$ and cytosine H_5 signals, leads to the conclusion that at least 90% of the

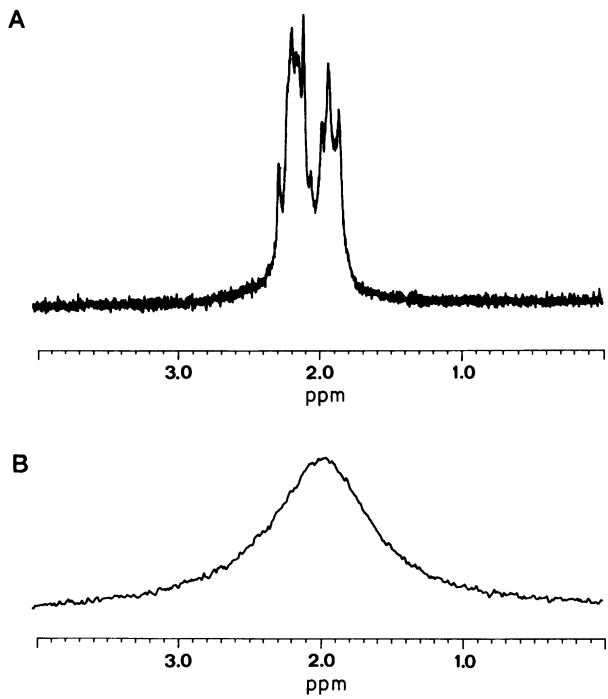

Figure 1. A. ^{31}P NMR spectrum of natural d(CAC.TCA.CCC.ATG.AAC.AGC). B. ^{31}P NMR spectrum of phosphate-methylated d(CAC.TCA.CCC.ATG.AAC.AGC).

phosphate groups are methylated. On the other hand, we suspect that the phosphates are not completely methylated, because during the deprotection of the exocyclic amino groups of the bases with triethylamine/pyridine (1:1) for 3 h some unwanted demethylation of the phosphates occurs. From a test with the phosphate-methylated dinucleotide d(TT) under the same conditions, it was established that approximately 5% of the phosphate groups had been demethylated after 3 h.

Furthermore, each phosphate-methylated oligodeoxynucleotide was characterized by means of UV hyperchromicity experiments. The UV absorbance as a function of temperature was monitored of a 1:1 mixture of phosphate-methylated and complementary natural DNA in an almost salt-free solution (no additional salts were added, $[Na^+] \approx 10^{-5}$ M). At this very low ionic strength short complementary natural DNA strands cannot form hybrids, due to the interstrand phosphate-phosphate repulsions (25,26). Duplexes of neutral phosphate-methylated DNA with natural systems, which lack electrostatic

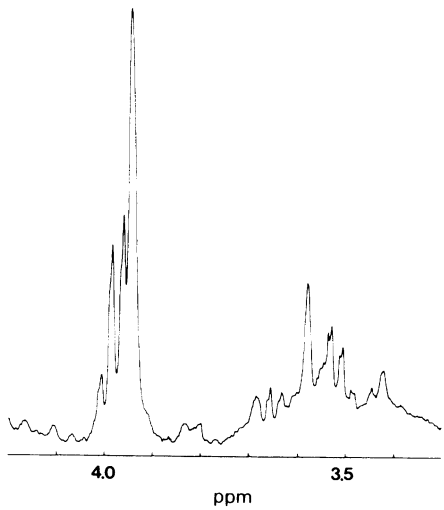

Figure 2. Subspectrum from the ^{1}H NMR spectrum of phosphate-methylated d(CAC.TCA.CCC.ATG.AAC.AGC).

interstrand repulsions, can be formed however. The melting temperatures (T_m values) that were obtained are shown in Table 1. The formation of very stable hybrids in almost salt-free solution clearly demonstrates the methylation of the phosphate groups. In all cases one sharp melting transition was observed, indicating that phosphorus chirality in the phosphate-methylated DNA does not

Table 1. Melting temperatures of 1:1 mixtures of a phosphate-methylated oligodeoxynucleotide with its natural complement; c_T = 2 μM, $[Na^+] \approx 10^{-5}$ M. Due to the high temperature, the T_m value of phosphate-methylated d(CAC.TCA.CCC.ATG.AAC.AGC) with its natural complement could not be determined exactly.

phosphate-methylated oligomer	Tm value (°C)
d(GGA.ATC)	27
d(AGC.CTG.AC)	42
d(GGA.ATC.CTG.CAG)	55
d(CAC.TCA.CCC.ATG.AAC.AGC)	70±5

influence the stability of hybrids with natural systems, in contrast with phosphate-ethylated DNA (10,13) and methyl phosphonate DNA (28). Using the nearest-neighbor method (28), the T_m values of 1:1 duplexes of methyl phosphonate and natural DNA were estimated at a total concentration of both the complementary oligodeoxynucleotides c_T = 2 μM: for methyl phosphonate d(GGA.ATC), d(AGC.CTG.AC), d(GGA.ATC.CTG.CAG) and d(CAC.TCA.CCC.ATG.AAC.AGC), -21 °C, 3 °C, 20 °C and 29 °C, respectively. So, compared with methyl phosphonate oligomers, very high T_m values are found for duplexes of phosphate-methylated DNA and natural DNA.

Stability of hybrids of phosphate-methylated and natural DNA

In order to investigate the stability of duplexes of phosphate-methylated DNA and natural DNA with respect to natural/natural hybrids, we have chosen the model system phosphate-methylated d(GGA.ATC.CTG.CAG)/natural d(CTG.CAG.GAT.TCC). For practical reasons (T_m values between 10 and 60 °C) a 12-mer/12-mer system was preferred. The T_m values of the neutral/natural and natural/natural duplexes were determined at various salt concentrations and are shown in Table 2. The T_m values of the natural/natural system at c_T = 2 μM were also calculated with the nearest-neighbor method (28); in 10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M, 10^{-1} M and 1 M NaCl, T_m values of -6 °C, 5 °C, 17 °C, 30 °C, 42 °C and 49 °C, respectively, were found. For natural/natural duplexes the T_m value increases with increasing ionic strength: better shielding of the negatively charged phosphate groups lowers the electrostatic interstrand repulsions. At low ionic strength ($[Na^+] = 10^{-5}$ M), no hybrid is formed, due to interstrand phosphate-phosphate repulsions. For neutral/natural duplexes on the other hand, the T_m value is high and (almost) independent of the ionic strength of the solution. This demonstrates that the electrostatic interstrand repulsions are effectively eliminated by phosphate-methylation in one strand.

Table 2. T_m values of natural d(GGA.ATC.CTG.CAG)/natural d(CTG.CAG.GAT.TCC) and phosphate-methylated d(GGA.ATC.CTG.CAG)/natural d(CTG.CAG.GAT.TCC) duplexes in 10^{-5} and 10^{-1} M NaCl; c_T = 2 μM.

$[Na^+]$	T_m value (°C) of the natural/natural duplex	T_m value (°C) of the neutral/natural duplex
≈ 10^{-5} M	< 5	55
10^{-1} M	42	56

For biological experiments T_m values obtained at 10^{-1} M NaCl are more significant than at 10^{-5} M of course. As the $[Na^+]$ increases, the difference in T_m value between neutral/natural and natural/natural DNA duplexes (ΔT_m) decreases, but at 10^{-1} M NaCl the ΔT_m of the 12-mer/12-mer system is still 14 °C.

When designing biological experiments it should be considered that the melting temperature is influenced by the total single strand DNA concentration (c_T). The total ss DNA concentration we used for the experiments to test the blocking effectiveness of phosphate-methylated oligodeoxynucleotides *in vitro* was approximately a factor 20 lower than for the UV experiments (0.1 and 2 μM, respectively). The decrease of the T_m value as a result of a 20-fold dilution was estimated by taking the value for natural systems: the melting temperature for natural/natural duplexes is approximately 10 °C lower at c_T = 0.1 μM with respect to c_T = 2 μM (25,26). For phosphate-methylated systems we expect a similar concentration-dependence of the T_m value. To be absolutely sure that the T_m value at c_T = 0.1 μM is sufficiently high for effective inhibition at room temperature, we have chosen a longer phosphate-methylated blocker for the *in vitro* experiments: the 18-mer d(CAC.TCA.CCC.ATG.AAC.AGC).

Effective and site-specific inhibition of DNA replication

Several *in vitro* systems have been used to study the effectiveness of antisense oligomers. Yet, with most methods it is difficult to demonstrate that the mechanism of inhibition is really an antisense mechanism. For phosphorothioates, for example, it is assumed that other mechanisms are also of importance, because at low concentrations phosphorothioates appear to inhibit HIV viral protein synthesis in a non-sequence-specific manner (7,8). Therefore we have developed an *in vitro* system to test antisense activity on the DNA replication level, based on the Sanger dideoxy sequencing technique. The intention was to demonstrate that an antisense phosphate-methylated oligodeoxynucleotide complementary to a region downstream of the primer can effectively block the sequencing pattern at that location. The *E.coli* pabB gene, inserted in the M13mp18 phage, was used as the template. Natural d(AGT.AAT.CAC.AGC.GGG.AGA), which is complementary to the 14 - 31 region of the pabB gene (27), was the primer for the sequencing reaction. The reaction was performed with the Klenow fragment of DNA polymerase I and standard deoxy/dideoxy ratios. Phosphate-methylated d(CAC.TCA.CCC.ATG.AAC.AGC), complementary to the -110 - -93 region of the gene, was used as site-specific antisense blocker. In order to ensure that phosphate-methylated DNA cannot act as a primer for Klenow DNA polymerase I, the sequencing procedure was

performed with the phosphate-methylated 18-mer, but without the natural primer. No sequencing pattern was observed. Hence, the Klenow fragment indeed does not recognize the neutral phosphate-methylated DNA as a primer for the polymerase reaction. The sequencing procedure, carried out with water instead of the phosphate-methylated octadecamer, was used as control experiment. The sequencing pattern with blocker (in a concentration of 3 μM) was far less intense downstream of the blocker than upstream, while the reference pattern was dark over the whole region. To quantify the blocking effectiveness we have measured the density of each band of the sequencing patterns optically. To eliminate the normally occurring density variation due to sequence-dependent strong and weak dideoxy-stops we have taken the ratio of the absorbance of the sequencing bands of the sequencing pattern with blocker and the corresponding bands of the reference pattern. The ratio as a function of location on the template is shown in Figure 3. This ratio can be interpreted as the relative synthesis activity along the template strand. The curve shows a sharp decrease of the ratio precisely at the position of the phosphate-methylated octadecamer. This observation demonstrates that a complementary phosphate-methylated 18-mer can effectively and specifically inhibit DNA replication via an antisense hybridization mechanism. A control experiment with the not

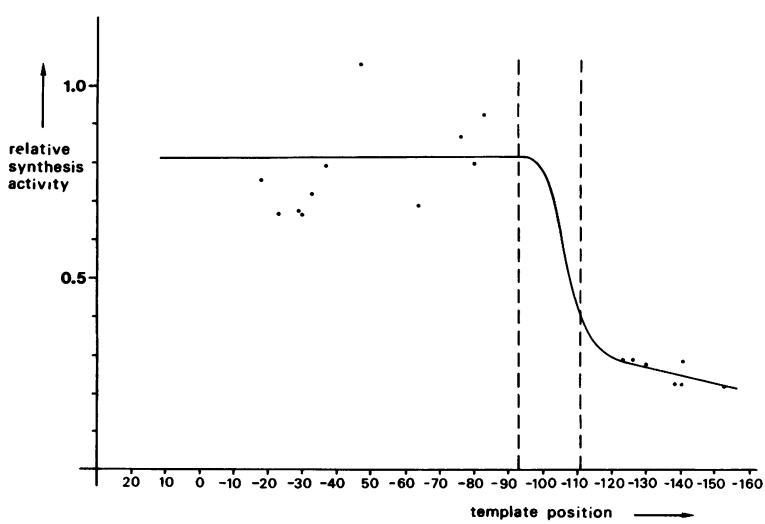

Figure 3. Relative synthesis activity along the template strand. The position of the complementary phosphate-methylated 18-mer is indicated with broken lines.

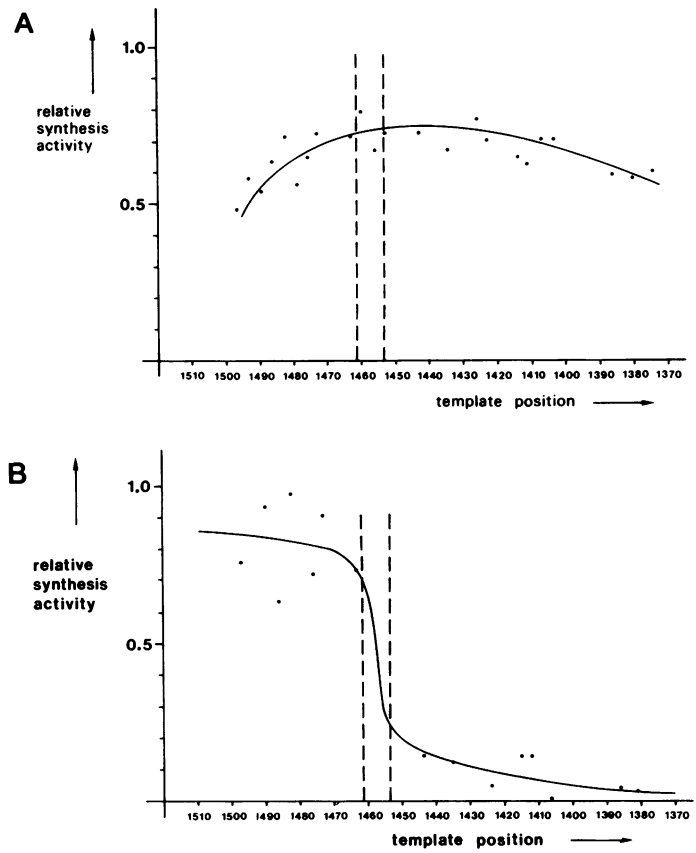

Figure 4. Relative synthesis activity along the template strand. The position of the complementary phosphate-methylated 8-mer is indicated with broken lines. A. Sequencing experiment performed at room temperature; no inhibition, due to insufficient hybridization of the phosphate-methylated 8-mer at room temperature. B. Sequencing experiment performed at 0 °C; inhibition of the DNA replication at the position of the phosphate-methylated 8-mer.

complementary phosphate-methylated 20-mer d(CTG.CTA.GAG.ATT.TTC.CAC.AC) showed no decrease of the relative synthesis activity and also no non-specific stops.

With the phosphate-methylated octamer d(AGC.CTG.AC), complementary to the 1455 - 1462 region of pabB, analogue blocker and control sequencing experiments were carried out. The resulting plot is given in Figure 4A. No decrease of relative synthesis activity is found downstream of the phosphate-methylated DNA. Obviously a phosphate-methylated octamer is too short to block the action of Klenow DNA Polymerase I effectively at room

temperature; the T_m value is not high enough for effective hybridization. By lowering the temperature, the hybridization is strengthened. When the same experiments were done at 0 °C, a normal sequencing pattern was observed until position 1463, where sequencing aborted, see Figure 4B. In each of the four lanes non-specific dark lines were seen precisely at the position of the phosphate-methylated octamer, caused by direct blocking of the DNA polymerase. From comparison of the experiments done at 22 °C and 0 °C it can be concluded that the T_m value of the phosphate-methylated/natural duplex is indeed an important parameter for antisense effectiveness: the phosphate-methylated octamer can inhibit the DNA replication process at 0 °C, but not at room temperature.

It would be interesting to compare the blocking effectiveness of the phosphate-methylated 18-mer with the 18-mer of the same sequence and unmodified phosphate groups. However, this natural 18-mer would act as a primer for the Klenow fragment too, so the sequencing procedure (*vide supra*) cannot be carried out. Unfortunately, it was not possible to prepare the 18-mer with a 3´-terminal dideoxynucleotide or with a 3'-terminal acetyl group, which are assumed not to be recognized by the Klenow fragment.

CONCLUSIONS

A new synthesis route has been developed to prepare phosphate-methylated oligodeoxynucleotides in a routine fashion. These neutral DNA compounds, which hybridize more strongly with single strand natural DNA than their natural analogues, can effectively inhibit the DNA replication process. For effective blocking at room temperature, a 18-mer is necessary. By using the Sanger dideoxy sequencing technique, it was shown that the blocking mechanism is site-specific.

REFERENCES

1. Marcus-Sekura, C.J. (1988) Anal. Biochem. 172, 289-295.
2. Walder, J. (1988) Genes & Development 2, 502-504.
3. Krol, A.R. van der, Mol, J.N.M. and Stuitje, A.R. (1988) BioTechniques 6, 958-976.
4. Zamecnik, P.C. and Stephenson, M.L. (1978) Proc. Natl. Acad. Sci. USA 75, 280-284.
5. Zon, G. (1988) Pharmaceutical Research 5, 539-549.
6. Sarin, P.S., Agrawal, S., Civeira, M.P., Goodchild, J., Ikeuchi, T. and Zamecnik, P.C. (1988) Proc. Natl. Acad. Sci. USA 85, 7448-7451.
7. Matsukura, M., Shinozuka, K., Zon, G., Mitsuya, H., Reitz, M., Cohen, J.S. and Broder, S. (1987) Proc. Natl. Acad. Sci. 84, 7706-7710.
8. Agrawal, S., Goodchild, J., Civeira, M.P., Thornton, A.H., Sarin, P.S. and Zamecnik, P.C. (1988) Proc. Natl. Acad. Sci. USA 85, 7079-7083.

9. Stein, C.A., Subasinghe, C., Shinozuka, K. and Cohen, J.S. (1988) Nucleic Acids Res. 16, 3209-3221.
10. Miller, P.S., Chandrasegaran, S., Dow, D.L., Pulford, S.M. and Kan, L.S. (1982) Biochem. 21, 5468-5474.
11. Koole, L.H., Genderen, M.H.P. van, Reiniers, R.G. and Buck, H.M. (1987) Proc. Kon. Ned. Akad. van Wetensch. B 90, 41-46.
12. Genderen, M.H.P. van, Koole, L.H., Merck, K.B., Meijer, E.M., Sluyterman, L.A.AE. and Buck, H.M. (1987) Proc. Kon. Ned. Akad. van Wetensch. B 90, 155-159.
13. Genderen, M.H.P. van, Koole, L.H. and Buck, H.M. (1989) Recl. Trav. Chim. Pays Bas, 108, 28-35.
14. Genderen, M.H.P. van, Koole, L.H. and Buck, H.M. (1988) Proc. Kon. Ned. Akad. van Wetensch. B 91, 53-55.
15. Genderen, M.H.P. van, Koole, L.H., Moody, H.M. and Buck, H.M. (1988) Proc. Kon. Ned. Akad. van Wetensch. B 91, 59-62.
16. Genderen, M.H.P. van, Koole, L.H. and Buck, H.M. (1988) Proc. Kon. Ned. Akad. van Wetensch. B 91, 179-183.
17. Miller, P.S. McParland, K.B., Jayaraman, K. and Ts'o, P.O.P. (1981) Biochem. 20, 1874-1880.
18. Miller, P.S., Agris, C.H., Blake, K.R., Murakami, A., Spitz, S.A., Reddy, P.M. and Ts'o, P.O.P. (1983) In Pullman, B. and Jortner, J. (eds), Nucleic Acids: The Vectors of Life, pp. 521-535, D. Reidel Publishing Company.
19. Blake, K.R., Murakami, A. and Miller, P.S. (1985) Biochem. 24, 6132-6138.
20. Koole, L.H., Quaedflieg, P.J.L.M., Kuijpers, W.H.A., Broeders, N.L.H.L., Langermans, H.A., Genderen, M.H.P. van, and Buck, H.M. (1988) Proc. Kon. Ned. Akad. van Wetensch. B 91, 205-209.
21. Koole, L.H., Broeders, N.L.H.L., Genderen, M.H.P. van, Quaedflieg, P.J.L.M., Wal, S.J. van der, and Buck, H.M. (1988) Proc. Kon. Ned. Akad. van Wetensch. B 91, 245-249.
22. Koole, L.H., Broeders, N.L.H.L., Quaedflieg, P.J.L.M., Kuijpers, W.H.A., Genderen, M.H.P. van, Coenen, A.J.J.M., Wal, S.J. van der, and Buck, H.M. (1989) J. Org. Chem., in press.
23. Miller, P.S., Fang, K.N., Kondo, N.S. and Ts'o, P.O.P. (1971) J. Am. Chem. Soc. 93, 6657-6665.
24. Sanger, F., Nicklen, S. and Couhon, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
25. Marky, A.M. and Breslauer, K.J. (1987) Biopolymers 26, 1601-1620.
26. Manning, G.S. (1978) Quarterly Reviews of Biophysics 11, 179-246.
27. Goncharoff, P. and Nichols, B.P. (1984) J. Bacteriol. 159, 57-62.
28. Quartin, R.S. and Wetmur, J.G. (1989) Biochemistry 28, 1040-1047.